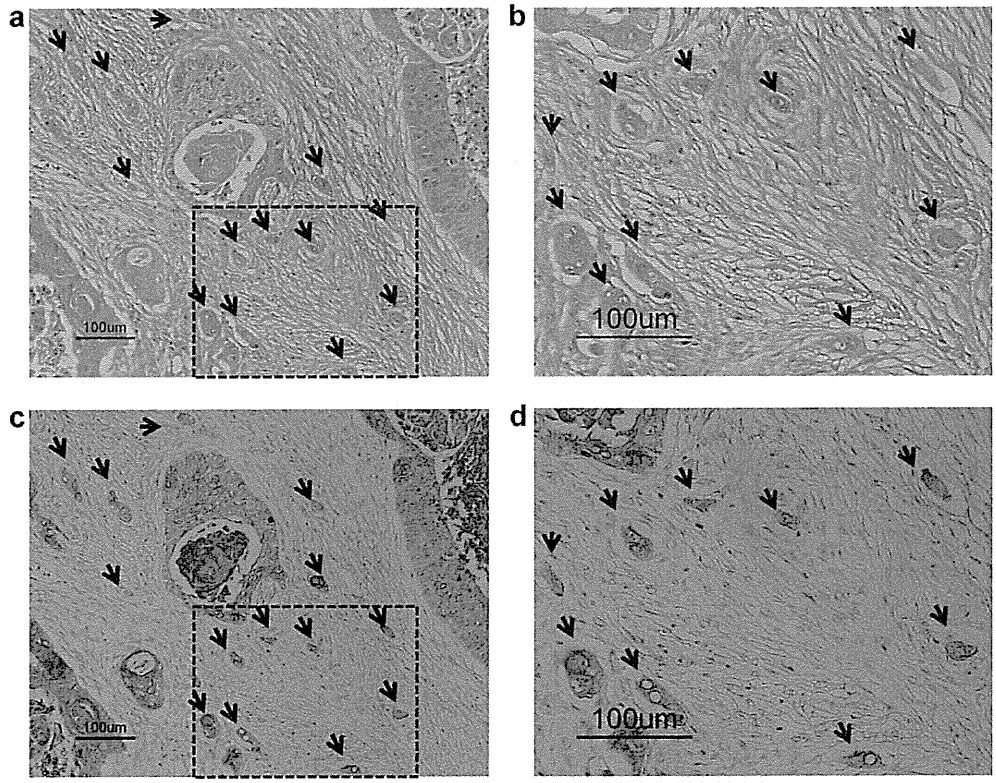


**Fig. 1.** Histological appearance of tumor budding. (a) Tumor budding in the area of fibrosis (hematoxylin and eosin [HE] staining). (b) High-power view of the area boxed in (a). Tumor budding is visible in the form of isolated small clusters or single cells with eosinophilic cytoplasm (HE staining). (c and d) Cytokeratin AE1/3 immunostaining of budding cells in same region as in (a) and (b).

significance of the budding process in SqCC. In addition, we elucidated the immunohistochemical characteristics of the budding cells (BCs) in the tumor specimens obtained from patients with SqCC-lung. Then, we examined the immunohistochemical profiles of budding cells to clarify their biological properties. Considering a characteristic morphological appearance of "budding cells", we used the following antibodies which had been reported being associated with cell adhesion (E-cadherin, β-catenin, CD44 and laminin-5γ2), hypoxia (carbonic anhydrase IX (CAIX) and GLUT-1), differentiation (podoplanin and p63), growth factor receptor (c-MET and IGF-1R), motility (ZEB-1 and MMP-7), and proliferation (Geminin) for immunohistochemistry.

## 2. Materials and methods

### 2.1. Patient selection

A total of 217 consecutive SqCC patients underwent complete tumor resection with a lobectomy or a more extensive surgery between January 2000 and December 2005 at the National Cancer Center Hospital East. Cases with history of neoadjuvant or adjuvant chemotherapy/radiotherapy were excluded. The median follow-up period of the patients was 4.7 years. All specimens were collected after obtaining written informed consent from the patients and the study was conducted with the approval of the Institutional Review Board of the National Cancer Center.

### 2.2. Histological studies

The surgical specimens were fixed in 10% formalin or 100% methyl alcohol. The primary tumor specimens were sliced at the maximum diameter and embedded in paraffin. Serial 4-μm-thick sections were stained with hematoxylin and eosin (HE) for light microscopy. Vascular and pleural invasion were evaluated in sections stained by the VVG method. The histological diagnoses were based on the revised World Health Organization (WHO) histological classification. Pathologic stage was determined based on the classification of the International Union Against Cancer (UICC-6) (Fig. 1).

### 2.3. Definition and evaluation of tumor budding

Isolated single cancer cells or clusters composed of lesser than five cancer cells in the stroma at the invasive margin of the tumor were defined as budding, as previously described [7]. For semi-quantitative assessment of the budding grade, the field in which the budding intensity appeared to be maximal was selected on the slide, and the number of budding cells/clusters in that field was counted under a 20× objective lens. Two observers (T.T. and G.I.) who had no knowledge of the clinical data independently reviewed the slides, and discordant results were resolved by a joint review of the specimen through a multiheaded conference microscope. Budding counts of 1–4 were rated as grade 1, of 5–10 as grade 2, and of 11 or more were as grade 3.

### 2.4. Antibodies and immunohistochemical staining

We selected 20 cases of SqCC-lung with grade 3 budding who had undergone surgery at our hospital between 2004 and 2010. The 13 antibodies used for immunohistochemical staining in this study are listed in the supplemental Table 1. Sections were cut from

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**Table 1**
Clinicopathological characteristics of the patients ($n$ = 217).

| Characteristics | All cases | Budding | | $p$ value |
|---|---|---|---|---|
| | | (−) (%) | (+) (%) | |
| No. of patients | 217 | 134 (61.8) | 83 (38.2) | |
| Age (year) | | | | |
| <70 | 105 | 64 (61.0) | 41 (39.0) | |
| ≥70 | 112 | 70 (62.5) | 42 (37.5) | 0.815 |
| Gender | | | | |
| Female | 15 | 11 (73.3) | 4 (26.7) | |
| Male | 202 | 123 (60.9) | 79 (39.1) | 0.339 |
| Smoking status | | | | |
| Nonsmoker | 3 | 2 (66.7) | 1 (33.3) | |
| Ever-smoker | 214 | 132 (61.7) | 82 (38.3) | 0.674 |
| Tumor size (mm) | | | | |
| ≤30 | 77 | 58 (75.3) | 19 (24.7) | |
| 30< | 140 | 76 (54.3) | 64 (45.7) | 0.002 |
| pN | | | | |
| pN0 | 139 | 104 (74.8) | 35 (25.2) | |
| pN1/pN2 | 78 | 30 (38.5) | 48 (61.5) | <0.001 |
| Differentiation | | | | |
| Well/moderate | 144 | 83 (57.6) | 61 (42.4) | |
| Poor | 73 | 51 (69.9) | 22 (30.1) | 0.08 |
| Pathological stage | | | | |
| IA | 63 | 52 (82.5) | 11 (17.5) | |
| IB | 71 | 48 (67.6) | 23 (32.4) | |
| IIA/IIB/IIIA | 83 | 34 (41.0) | 49 (59.0) | <0.001 |
| Vascular invasion | | | | |
| Absent | 73 | 64 (87.7) | 9 (12.3) | |
| Present | 144 | 70 (48.6) | 74 (51.6) | <0.001 |
| Lymphatic permeation | | | | |
| Absent | 164 | 113 (68.9) | 51 (31.1) | |
| Present | 53 | 21 (39.6) | 32 (60.4) | <0.001 |
| Pleural invasion | | | | |
| Absent | 135 | 91 (67.4) | 44 (32.6) | |
| Present | 82 | 43 (52.4) | 39 (47.6) | 0.028 |
| Tumor location | | | | |
| Central | 87 | 43 (49.4) | 44 (50.6) | |
| Peripheral | 130 | 91 (70.0) | 39 (30.0) | 0.002 |

the paraffin blocks and mounted on silanized slides, deparaffinized in xylene, and dehydrated in a graded ethanol series. They were then washed three times in distilled water and phosphate-buffered saline (PBS) and placed in 0.1 M of citric acid buffer (pH 6.0). Antigen retrieval was performed by heating the slides to 95 °C for 20 min in a microwave oven (H2800 Microwave Processor, Energy Beam Sciences Inc.). For laminin-5γ2 antigen retrieval, sections were digested with Proteinase K (Dako, Glostrup, Denmark) for 10 min at room temperature. Next, the slides were washed three times in PBS and immersed in a 0.3% hydrogen peroxide solution in methanol for 15 min to quench endogenous peroxidase activity. Non-specific binding was blocked by preincubation with 2% BSA plus 0.1% $NaN_3$ for 30 min. The slides were incubated overnight at 4 °C with the primary antibodies listed in the supplemental table. The slides were washed again three times with PBS and incubated with the EnVision + System HRP (Dako) for 60 min. The chromogen used was 2% 3,3′-diaminobenzidine in 50 mM Tris buffer (pH 7.6) containing 0.3% hydrogen. Finally, the slides were counterstained with Meyer's hematoxylin, dehydrated, and mounted. We used positive control of each antibody in the immunohistochemical study as follows: E-cadherin, β-catenin, CD44, c-MET and IGF-1R: bronchial epithelium, laminin-5γ2: basement membrane of bronchial epithelium, CAIX: renal cell carcinoma, GLUT-1; red blood cell, podoplanin and p63; basal cell of bronchial epithelium, MMP-7: human lung adenocarcinoma, ZEB-1; stromal fibroblast, Geminin: lymphocytes in germinal center. We confirmed that positive control tissues were stained by each antibody, and we also performed negative control studies that were made without primary antigen in all antibodies.

### 2.5. Calculation of the staining scores

All the stained tissue sections were semi-quantitatively scored and evaluated independently under a light microscope by two observers (T.T. and G.I.) with no knowledge of the clinicopathological data. When their evaluations differed, the tissue was examined by both through a multiheaded conference microscope and a consensus was reached. The labeling scores, except for Geminin, were calculated by multiplying the percentage of positively stained tumor cells per lesion (0–100%) by the staining intensity level (0: negative, 1: weak and 2: strong) and the scores ranged from 0 to 200. In the case of Geminin, positively stained tumor cells per 100 tumor cells were counted and the scores ranged from 0 to 100.

### 2.6. Statistical analysis

The differences in the clinicopathological features between the budding (+) group and budding (−) group were evaluated by the Chi-square test or Fisher's exact test, as appropriate. Overall survival was defined as the time from the date of surgery to the date of death from any cause, or the last date on which the patient was known to be alive. Univariate analyses were conducted by the Kaplan–Meier method, and the statistical significance of the differences between the survival curves was assessed by the log-rank test. To assess the independent effects of the different variables on survival, multivariate analyses were carried out using a Cox proportional hazards model. Only variables that were found to be significant in the univariate analyses were entered into the stepwise backward Cox regression model. The probabilities of stepwise

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entry and removal were set at 0.05 and 0.10, respectively. The Mann–Whitney's *U* test was used to compare the staining scores. *p* values of <0.05 were considered to denote statistical significance. Two-sided statistical tests were used for all the analyses, except for Fisher's exact test, for which a one-sided statistical test was employed. All statistical analyses were performed using SPSS II for Windows, version 11.0.1J.

## 3. Results

### 3.1. Clinicopathological findings

The clinicopathological data of the 217 patients of SqCC-lung are listed in Table 1. Tumor budding was observed in 83 (38.2%) cases. The median age of the patients was 70 years (range: 44–88), and the median tumor size was 3.8 cm (range: 1.0–12.0). Presence of tumor budding was significantly correlated with a larger tumor size ($p = 0.002$), presence of lymph node metastasis ($p < 0.001$), advanced pathologic stage ($p < 0.001$), and presence of vascular invasion ($p < 0.001$), lymphatic permeation ($p < 0.001$) and pleural invasion ($p = 0.028$) (Table 1).

### 3.2. Prognostic significance of tumor budding

The overall survival rate of the budding (+) group was significantly lower than that of the budding (−) group ($p = 0.001$, Fig. 2a), and the 5-year survival rates were 45.6% and 64.0%, respectively. The disease specific survival rate of the budding (+) group was also significantly lower than that of the budding (−) group ($p < 0.001$, Fig. 2b). Analysis according to the grade of tumor budding (grades 1–3) showed that the outcomes in all of the groups with grade 1, grade 2 and grade 3 budding were significantly poorer than the outcome in the group without tumor budding (grade 0) ($p = 0.031$ and $p = 0.026$, $p = 0.002$, respectively) (Fig. 2c). The presence tumor budding was found to be predictive of reduced overall survival in patients with pathological stage IA ($p = 0.021$) (Supplemental Fig. a). By contrast, there were no significant differences in the overall survival between cases with and without budding in patients with pathological stage IB or II + III disease ($p = 0.84$ and $p = 0.089$, respectively) (Supplemental Fig. b and c).

### 3.3. Univariate and multivariate analysis

Univariate analysis was performed to determine the prognostic significance of budding (Supplemental Table 2). The presence of tumor budding was significantly correlated with a shorter survival time ($p = 0.001$). In addition, a larger tumor size ($p = 0.031$), and the presence of lymph node metastasis ($p = 0.002$), lymphatic permeation ($p = 0.041$), vascular invasion ($p = 0.034$), and pleural invasion ($p < 0.001$) were all correlated with a shorter survival time (Supplemental Table 2). Multivariate analysis using a Cox proportional hazard model was performed using stepwise backward selection for the following factors that were found to be significant ($p < 0.05$) by univariate analysis: tumor size, and presence/absence of lymph node metastasis, lymphatic permeation, vascular invasion, pleural invasion, and budding. The variables that were not significant were removed by analysis of software, and last analysis was performed with three significant variables (lymph node metastasis, pleural invasion and budding). Presence of tumor budding ($p = 0.022$) and pleural invasion ($p = 0.008$) were finally identified as significant independent prognostic factors (Table 2).

### 3.4. Immunohistochemical profiles of budding cells

We evaluated the immunohistochemical profiles of the budding cells by staining of tumor cells at two sites: (1) budding cells and (2) the tumor cells forming solid nest nearest to the budding cells (Fig. 3). Comparisons of the staining scores between the two sites are summarized in Fig. 4 according to the antibody used.

#### 3.4.1. Expressions of cellular adhesion molecules

The median staining scores for E-cadherin in the budding cells and tumor cells forming solid nests were 10 and 50, respectively. The median staining score for β-catenin in the budding cells and tumor cells forming solid nests were 90 and 150, respectively. The mean staining scores for CD44 in the budding cells and tumor cells forming solid nests were 145 and 160, respectively. The E-cadherin, β-catenin and CD44 expression levels in the budding cells were significantly lower than those in the tumor cells forming solid nests (E-cadherin: $p = 0.004$; β-catenin: $p = 0.002$; CD44: $p = 0.034$) (Fig. 4). The median staining scores for laminin-5γ2 in the budding cells and tumor cells forming solid nests were 140 and 60, respectively The laminin-5γ2 expression level in the budding cells was significantly higher than that in the tumor cells forming solid nests ($p = 0.001$) (Fig. 4).

#### 3.4.2. Expression of hypoxia-induced protein

The median staining scores for CAIX in the budding cells and tumor cells forming solid nests were 5 and 35, respectively. The median staining scores for GLUT-1 in the budding cells and tumor cells forming solid nests were 40 and 110, respectively. The CAIX and GLUT-1 expression levels in the budding cells were significantly lower than those in the tumor cells forming solid nests (CAIX: $p = 0.023$; GLUT-1: $p < 0.001$) (Fig. 4).

#### 3.4.3. Expression levels of the differentiation markers

The median staining scores for podoplanin in the budding cells and tumor cells forming solid nests were 85 and 75, respectively. The median staining scores for p63 in the budding cells and tumor cells forming solid nests were 135 and 145, respectively. There were no significant differences in the expression levels of podoplanin or p63 expression in the cancer cells according to the site (Fig. 4).

#### 3.4.4. Expression level of the growth factor receptor

The median staining scores for IGF-1R in the budding cells and tumor cells forming solid nest were 50 and 75, respectively. The median staining scores for c-MET in the budding cells and tumor cells forming solid nests were 50 and 50, respectively. There were no significant differences in the expression levels of IGF-1R and c-MET expression in the cancer cells according to the site (Fig. 4).

#### 3.4.5. Others

The median staining scores for ZEB-1 in the budding cells and tumor cells forming solid nests were 0 and 0, respectively. The median staining scores for MMP-7 in the budding cells and tumor cells forming solid nests were 0 and 0, respectively. There were no significant differences in the expression levels of ZEB-1 and MMP-7 in the cancer cells according to the site (Fig. 4). Significantly fewer Geminin-positive cells per 100 tumor cells were detected in the budding cells than in tumor cells forming solid nests (median: 15 vs. 29; $p = 0.008$) (Fig. 4).

## 4. Discussion

This is the first study to evaluate the prognostic significance of tumor budding and its biological characteristics in SqCC of the lung. Similar to reports on tumor budding in adenocarcinoma, our data showed that tumor budding in SqCC-lung was associated with several prognostic factors: tumor size, presence/absence of lymph node metastasis, pathologic stage, presence/absence of vascular invasion, lymphatic permeation, and pleural invasion [10–12]. In addition, presence of pleural invasion and tumor budding were

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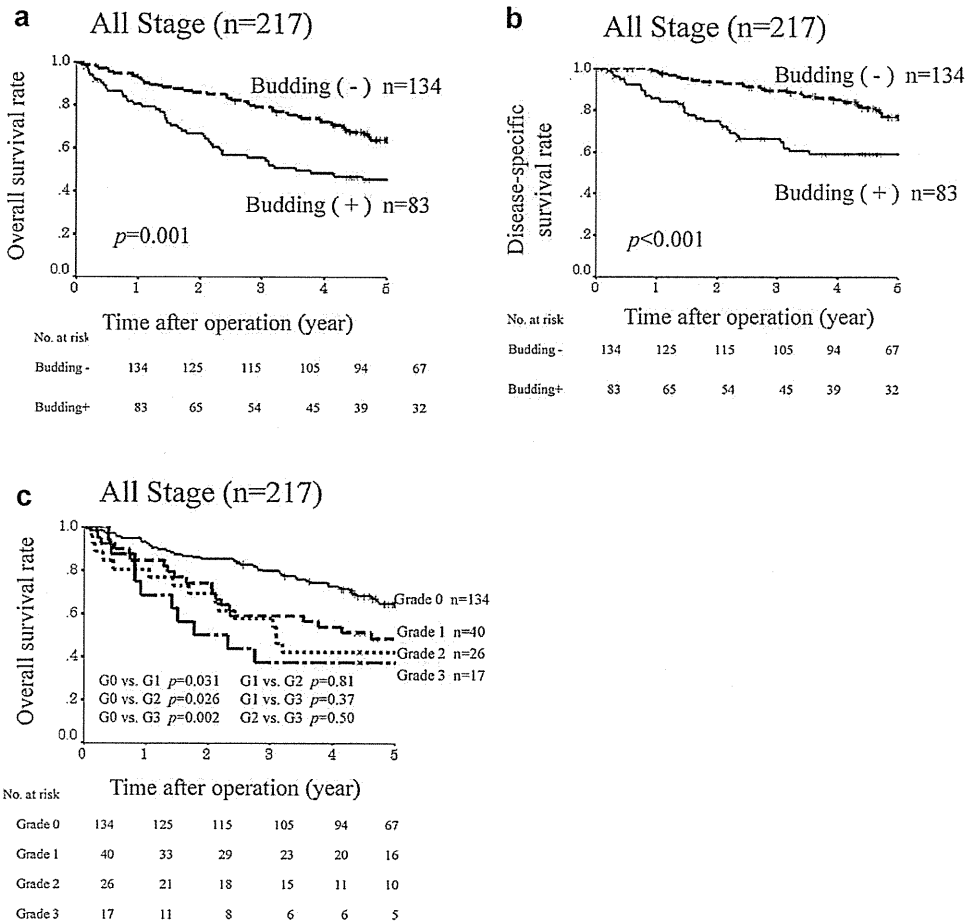


**Fig. 2.** Cumulative overall survival curves. (a) Cumulative overall survival curves of 217 patients stratified by the presence/absence of tumor budding. (b) Cumulative disease-specific survival curves of 217 patients stratified by the presence/ absence of tumor budding. (c) Cumulative overall survival curves of 217 patients stratified by the grade of tumor budding.

identified by multivariate analysis as statistically significant factors influencing the overall survival. Maeshima et al. reported that single cell invasion is a predictor of poor prognosis in patients with small-sized SqCC of the peripheral lung [13]. These findings were consistent with our data, and we can consider that the presence of tumor budding is a universal poor-prognostic factor, irrespective of the histological type or size.

Our current findings reveal that BCs exhibit reduced membranous expression levels of the cellular adhesion molecules E-cadherin, and β-catenin, and increased expression levels of laminin-5γ2, compatible with the budding phenotype of adenocarcinoma reported previously [14,15]. According to recent molecular research, several reports have revealed that tumor budding is closely related to EMT [16,17]. EMT has been recognized as the phenomenon by which tumor cells in primary lesions invade the surrounding stroma [18]. Tumor progression is generally considered to involve spatial and temporal occurrences of EMT, whereby tumor cells acquire a more invasive and metastatic phenotype [19]. Geminin, which is expressed during the S, G2 and early M phase, is also a protein which plays a crucial role in the regulation of the cell cycle [20]. It is accepted as a cell proliferation marker in normal tissues as well as malignant tissues [21]. The finding that BCs expressed a lower level of Geminin in comparison with tumor cells forming solid nests is consistent with previous reports that increased expression of laminin-5 and decreased expression of the E-cadherin–β-catenin complex, which exhibits the EMT phenotype, is linked focally to a non-proliferating status of the budding tumor cells [19,22]. Our current findings suggest that BCs in

**Table 2**
Multivariate analysis for overall survival ($N = 217$).

| Variables | Favorable | Unfavorable | Hazard ratio [95% CI] | $p$-Value |
|---|---|---|---|---|
| Tumor size | ≤30 | >30 | – | – |
| $N$ stage | pN0 | pN1/pN2 | 1.419 [0.939–2.142] | 0.096 |
| Lymphatic permeation | Absent | Present | – | – |
| Vascular invasion | Absent | Present | – | – |
| Pleural invasion | Absent | Present | 1.717 [1.155–2.552] | 0.008 |
| Budding | Absent | Present | 1.597 [1.069–2.384] | 0.022 |

CI: confidence interval.

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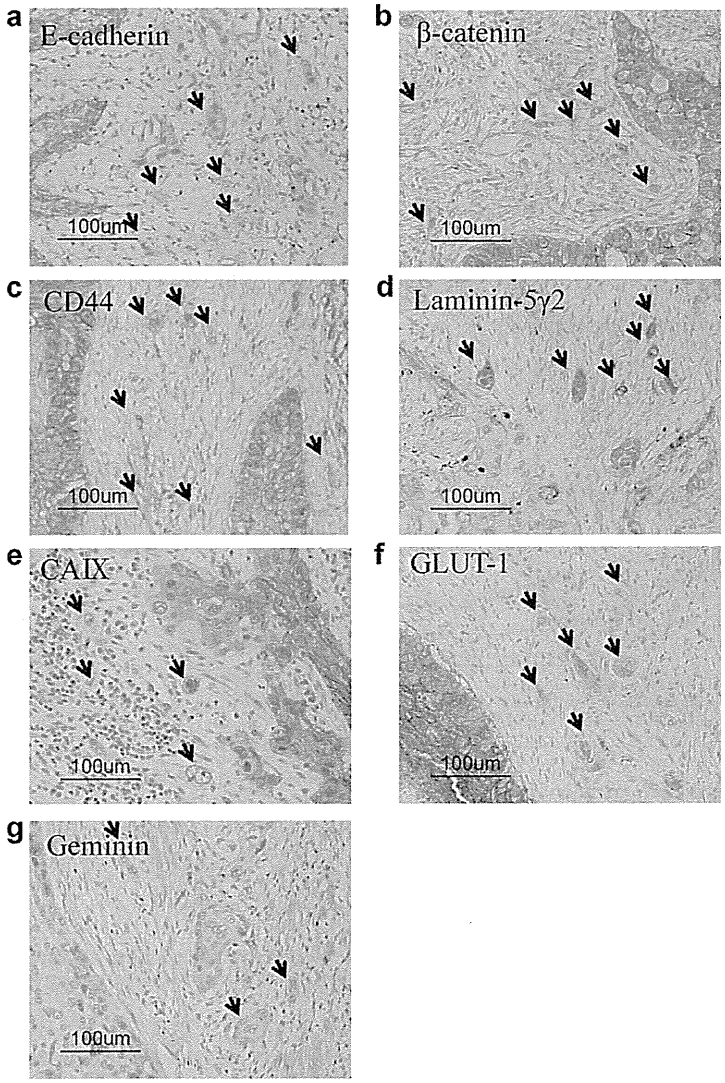


**Fig. 3.** Immunohistochemical staining (a) E-cadherin staining: decreased membrane immunostaining was found in BCs (arrows) as compared with that in the tumor cells forming solid nests. (b) β-Catenin staining: decreased membrane immunostaining was found in BCs (arrows) as compared with that in the tumor cells forming solid nests. (c) CD44 staining: decreased cytoplasm and membrane immunostaining was found in BC (arrows) as compared with that in the tumor cells forming solid nests. (d) Laminin-5γ2 staining: increased cytoplasm immunostaining was found in BC (arrows) as compared with that in the tumor cells forming solid nests. (e) CAIX staining: decreased membrane immunostaining was found in BC (arrows) as compared with that in the tumor cells forming solid nests. (f) GLUT-1 staining: decreased cytoplasm immunostaining was found in BC (arrows) as compared with that in the tumor cells forming solid nests. (g) Geminin staining: decreased nuclei immunostaining was found in BC (arrows) as compared with that in the tumor cells forming solid nests.

SqCC-lung also exhibit the display EMT phenotype, which may be one of the reasons why the presence of tumor budding is also a poor-prognostic factor in SqCC-lung.

Comparison of the immunohistochemical staining profiles between SqCC and adenocarcinoma revealed that while the expression patterns of the EMT marker were the same, those of the differentiation and hypoxia markers were different. No significant differences in the expression levels of the differentiation markers podoplanin and p63 were found between the budding cells and tumor cells forming solid nests in SqCC-lung. These findings are inconsistent with a previous report that the BCs in adenocarcinoma have undergone dedifferentiation [7,23,24]. The BCs in colorectal cancer undergo dedifferentiation just like those in lung adenocarcinoma, however, expression of a differentiation marker in BCs, like the observation in SqCC-lung, is unprecedented. In the current study, we examined two differentiation markers, podoplanin and p63, and found that the expressions of these markers on the BCs were not different from those on the cancer cells forming nests. Therefore, it may be reasonable to speculate that the absence of dedifferentiation of the BCs may be a distinctive characteristic of SqCC-lung.

In regard to the expression of the hypoxia marker, the expression level was significantly lower in BCs than in the tumor cells forming solid nests in SqCC-lung. On the other hand, no significant difference in the expression level of the hypoxia marker was reported between the BCs and tumor cells forming solid nests in adenocarcinoma. Two reasons may be put forth to explain this finding. First, SqCC is known to be more necrotic than other histologic subtypes of NSCLC, suggesting that the former may be characterized by a more hypoxic environment [25]. Secondly, adenocarcinoma of

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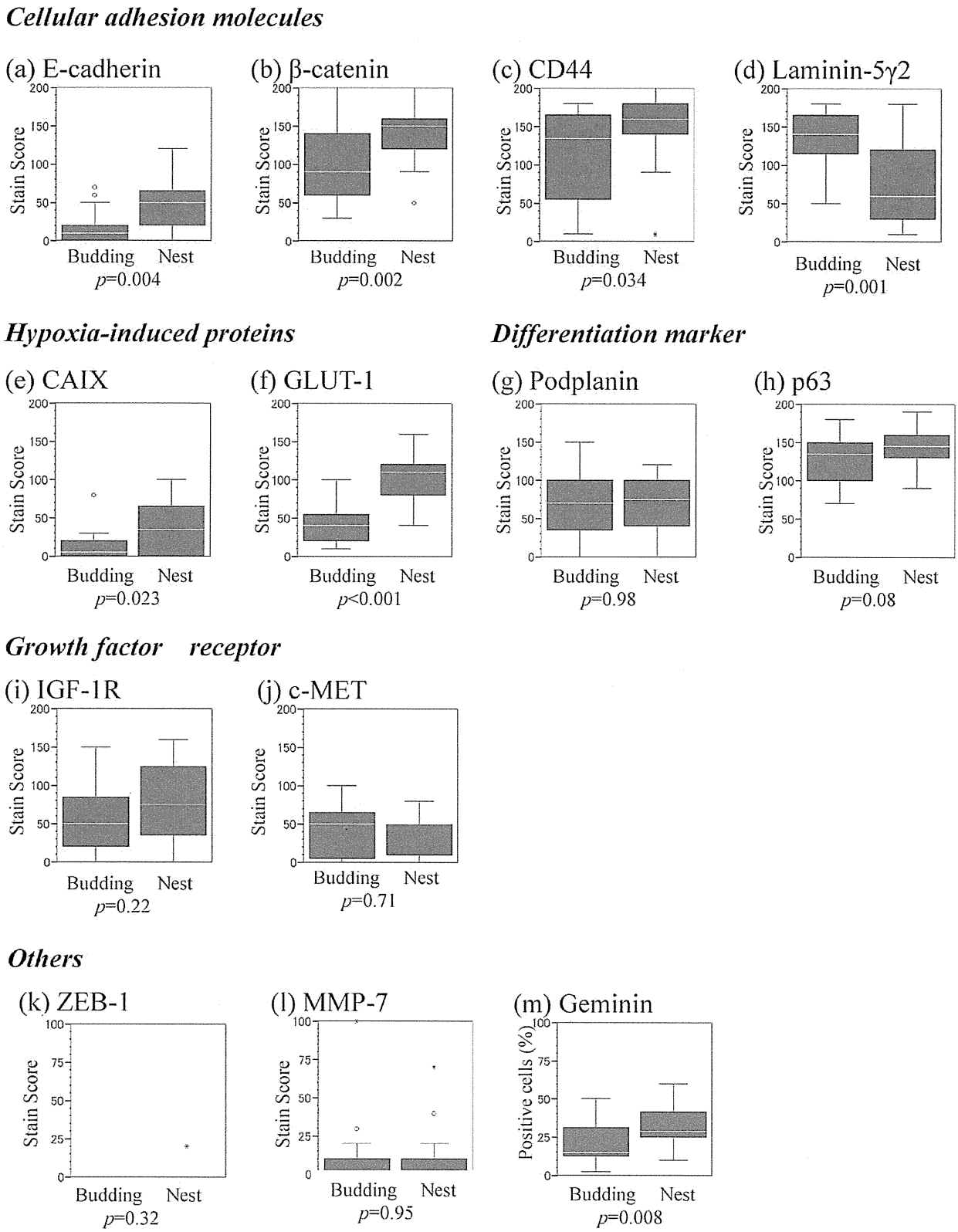


**Fig. 4.** Comparison between the immunohistochemical scores of budding cells and tumor cells forming solid nests. Central box, values from the lower to upper quartile (25–75 percentile). In the box plots, the middle line represents the median. The whisker extends from the minimum to the maximum value. Outliers are plotted with a round marker. (a) E-cadherin, (b) β-catenin, (c) CD44, (d) laminin-5γ2, (e) CAIX, (f) GLUT-1, (g) podoplanin, (h) p63, (i) IGF-1R, (j) c-MET, (k) ZEB-1, (l) MMP-7 and (m) Geminin.

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the lung is well known to show histological heterogeneity reflecting its biological diversity, and CAIX expression in lung adenocarcinoma may be regulated by molecular mechanisms other than tumor hypoxia [26].

As for the prognosis, significant difference was found only in the groups with higher grades of budding (grades 2 and 3) in lung adenocarcinoma [7]. On the other hand, in SqCC-lung, the outcome in budding (+) cases was poor regardless of the budding grade. Prognostic evaluation according to the disease stage revealed that the outcome was significantly poorer in budding (+) cases in patients with stage IA disease, but no significant difference was noted between the budding (+) and budding (−) cases among patients with other disease stages. Thus, it is thought that the influence of other poor-prognostic factors might become more and more predominant as the disease stage advances.

In conclusion, the results of this study demonstrated that tumor budding is an important predictor of poor prognosis in patients with SqCC of the lung who have undergone complete resection. The results also suggest that BCs in SqCC-lung exhibit the EMT phenotype in part. However, the biological characteristics of budding cells in SqCC-lung differ, at least in part, from those of the budding cells in lung adenocarcinoma. Further analysis of the biological characteristics of budding in SqCC-lung might pave the way for the development of new treatment strategies for SqCC-lung.

## Conflict of interest

The authors declare no conflict of interest.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.lungcan.2011.11.010.

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Please cite this article in press as: Taira T, et al. Characterization of the immunophenotype of the tumor budding and its prognostic implications in squamous cell carcinoma of the lung. Lung Cancer (2011), doi:10.1016/j.lungcan.2011.11.010

# Risk Factors for Treatment-Related Death Associated with Chemotherapy and Thoracic Radiotherapy for Lung Cancer

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**Introduction:** The aim of the study is to evaluate the current status of treatment-related death (TRD) in lung cancer patients.

**Methods:** We retrospectively analyzed the incidence and risk factors of TRD in lung cancer patients who received chemotherapy and/or thoracic radiotherapy using logistic regression analyses.

**Results:** Between January 2001 and December 2005, 1225 (222 small cell and 1003 non-small cell lung cancers) patients received chemotherapy and/or thoracic radiotherapy as the initial treatment. Of these, 43 patients receiving chemotherapy followed by thoracic radiotherapy were included into both the chemotherapy-alone and radiotherapy-alone groups. There were a total of 23 (1.9%) TRDs. Chemotherapy-related deaths occurred in 7 of 927 (0.8%) patients, including 4 from drug-induced lung injury, 2 from pneumonia, and 1 from unknown cause. Concurrent chemoradiotherapy-related deaths occurred in 12 of 245 (4.9%) patients, including 11 from radiation pneumonitis and 1 from pneumonia. Thoracic radiotherapy-related deaths occurred in 4 of 96 (4.2%) patients. The incidence of chemotherapy-related death was correlated with poor performance status (odds ratio [OR]: 11.4, 95% confidence interval [CI]: 3.53–37.1), the presence of hypoxia (OR: 19.3, CI: 6.06–61.7), hyponatremia (OR: 45.5, CI: 13.4–154), and treatment with epidermal growth factor receptor-tyrosine kinase inhibitors (OR: 8.56, CI: 2.48–29.5), whereas the incidence of concurrent chemoradiotherapy-related death was correlated with pulmonary fibrosis (OR: 22.2, CI: 5.61–87.8). Radiotherapy results were not analyzed because there were too few patients.

**Conclusions:** TRD occurred in 1.9% of the patients as a result of treatment-related lung injury in the majority of the cases.

**Key Words:** Lung cancer, Treatment-related death, Risk factor, Chemotherapy, Thoracic radiotherapy.

(*J Thorac Oncol.* 2012;7: 177–182)

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Disclosure: The authors declare no conflict of interests.

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ISSN: 1556-0864/12/0701-0177

Before any medical interventions are undertaken in patients with lung cancer, they must be clearly informed about the risks and benefits of the intervention(s) and about alternative treatment options. Careful delivery of this is particularly important if the planned treatment may not only result in cure but may also be harmful. Provision of accurate information to help patients make the most appropriate decision is therefore crucial. However, the risks of death from drug toxicity and the incidences of such events tend to be uncertain[1–4] and also constantly change with the wide use of newer agents, such as third-generation chemotherapy agents, and molecular-targeted agents. In addition, the incidence of treatment-related deaths (TRDs) has not been thoroughly examined in clinical settings outside of clinical trials. Prospective clinical trials for poor-risk patients are often difficult to perform because of poor accrual, reflecting the reluctance of physicians to subject patients with underlying comorbid illness to the toxic effects of chemotherapy and radiation.

Our ultimate goal is to prospectively identify individuals who are at a high risk of TRD so as to provide the most precise estimation of the possible risks to each patient. In this study, we retrospectively examined the data of patients with locally advanced or metastatic lung cancer who were treated at the National Cancer Center Hospital, Tokyo, Japan, focusing on the risks and incidences of TRD associated with chemotherapy and radiotherapy.

## PATIENTS AND METHODS

### Patients

Between January 2001 and December 2005, a total of 1623 lung cancer patients were admitted to the thoracic oncology ward at the National Cancer Center Hospital. All patients were admitted in this period to be treated as part of standard practice in Japan. Patients who received chemotherapy alone usually stayed in the hospital for 7 to 10 days for one cycle of chemotherapy, and those who received concurrent chemoradiotherapy stayed for 6 weeks. Among these, a total of 1225 patients who had received first-line chemotherapy and/or radiotherapy on an inpatient basis were extracted from the institutional database. Additional details about the patients, including the diagnostic imaging findings, were then reviewed from the patients' medical records. The data of patients receiving chemotherapy and/or thoracic radiotherapy

as the initial treatment were evaluated. They included patients with stage III to IV disease and postoperative recurrent disease who received chemotherapy; those with stage III disease who received chemoradiotherapy or radiotherapy alone; and those with stage III disease who received preoperative induction therapy or postoperative adjuvant therapy. All the patients had been followed for at least 4 weeks after the completion of treatment.

## Treatment Selection

After a thorough evaluation of the operability and/or curability, the eligibility of each patient for enrollment in an open clinical trial was determined. Although patient recruitment for protocol treatments is a priority of ours, patients were free to refuse treatment. If no appropriate clinical trials were scheduled or under way, the known best standard treatments were administered.

## Best Standard Treatments

For first-line treatment, patients with non-small cell lung cancer (NSCLC) who were deemed inoperable but curable with good local control with chemoradiotherapy received three to four cycles of cisplatin (CDDP) 80 mg/m$^2$ on day 1 + vinorelbine (VNR) 20 mg/m$^2$ on days 1 and 8, every 4 weeks, along with early concurrent thoracic radiotherapy, usually at a total dose of 60 Gy/30 fractions.[5] Sequential chemoradiotherapy, rather than concurrent chemoradiotherapy, was offered if the calculated percentage of the total lung volume receiving radiation in excess of 20 Gy ($V_{20}$) was more than 40%.[6] Thoracic radiotherapy alone was selected if chemotherapy could not be given due to comorbidity. If the radiation field involved the contralateral hilum or if the patients had malignant effusion and/or distant metastasis, platinum doublet therapy was administered; the most common combination was four cycles of carboplatin (CBDCA) area under the curve = 6 on day 1 + paclitaxel (PTX) 200 mg/m$^2$ on day 1, every 3 weeks.[7] For limited-disease SCLC, four cycles of a combination of CDDP 80 mg/m$^2$ on day 1 + etoposide 100 mg/m$^2$ on days 1 to 3, every 4 weeks, were administered concurrently with hyperfractionated thoracic radiotherapy at a total radiation dose of 45 Gy in fractional doses of 1.5 Gy, administered twice a day.[8] In patients with extensive-disease SCLC, four cycles of a combination of CDDP 60 mg/m$^2$ on day 1 and irinotecan (CPT) 60 mg/m$^2$ on days 1, 8, and 15, every 4 weeks, were usually administered.[9] Radiotherapy was given using megavoltage photons (6–15 MV). The routine radiation schedule without chemotherapy for locally advanced NSCLC was a total radiation dose of 60 to 66 Gy, or as high as 70 Gy, administered in fractional doses of 2.0 Gy once a day.

## Definition of TRD

Chemotherapy-related death was defined as death occurring within 4 weeks of the completion of treatment, without clear evidence of any other cause of death, or death obviously caused by treatment toxicity. Radiotherapy-related death was defined as death secondary to hypoxia or to complications of corticosteroid administration after the diagnosis of radiation pneumonitis. Steroid therapy was administered based on the attending physician's discretion, without a standardized treatment dose or duration, for the management of radiation-induced lung injury.[10]

## Definition of Treatment-Induced Lung Injury

The criteria of drug-induced lung injury in this study were as follows: (1) appearance of new symptoms and radiological abnormalities in the course of chemotherapy with the onset within a few months of the start of the therapy; (2) diffuse or multifocal ground-glass opacities and intralobular interstitial thickening without segmental distribution in computed tomography (CT) scans of the chest; and (3) no evidence of underlying heart disease, infection, or lymphangitic carcinomatosis. Lung biopsy was not routinely performed in our hospital because patients were frequently too frail to undergo biopsy. The criteria of radiation-induced lung injury were (1) appearance of new symptoms and radiological abnormalities with the onset within 6 months of the end of thoracic radiotherapy; (2) opacification, diffuse haziness, infiltrates, or consolidation conforming to the outline of the sharply demarcated irradiated area in CT scans; and (3) a reduction in lung volume within the irradiated area and linear, ground-glass opacities or reticular shadows beyond the irradiated area developing during clinical course. In contrast, the criteria of bacterial pneumonia were (1) clinical suspicion of pneumonia including rapidly developing fever and/or productive cough; and (2) consolidation spreading through anatomical structure of the lung in CT scans.

## Statistical Analysis

We investigated the associations between chemotherapy-related or concurrent chemoradiotherapy-related death and the potential risk factors at the time of diagnosis. The following potential risk factors were investigated: sex, age (≥70 years versus <70 years), performance status (Eastern Cooperative Oncology Group criteria; 2–4 versus 0–1), smoking history (presence versus absence), partial pressure of oxygen (70 mmHg ≤ $PO_2$ versus >70 mmHg), hemoglobin (Hgb < 13.7 g/dl versus ≥ 13.7 g/dl), platelet (Plt > 367 × $10^9$/L versus ≤367 × $10^9$/L), albumin (Alb < 3.7 g/dl versus ≥3.7 g/dl), sodium (Na < 138 mEq/L versus ≥138 mEq/L), clinical trial (in versus out), and chemotherapy regimen (The cutoff values of hemoglobin, platelet, albumin, and sodium are the institutional normal limits [above or below]). For concurrent chemoradiotherapy-related factors, the presence of coincidental diseases such as emphysema (with versus without) or pulmonary fibrosis (with versus without) and the location of the primary tumor (lower lobe versus other lobes) were also included in the analyses. The diagnostic criteria of pulmonary fibrosis were a linear, ground-glass attenuation or reticular shadows on chest radiographs and CT scans before treatment that were predominant in the lower zone of the lung. Also, the influence of the chemotherapy regimens was evaluated.

In the univariate preliminary analysis, the relation between previously defined variables at the time of presentation and the occurrence of the outcome variable (toxic death) was assessed using the $\chi^2$ test. To adjust for each factor, multivariate logistic regression analyses were planned. When the number of observed events was less than 10, multivariate

analysis was not performed. When the number of patients for each factor was small, the factor was excluded from the model, even when it appeared to be statistically significant. All the analyses were performed using the STATISTICA 4.1J program (StatSoft, Inc., Tulsa, OK).

## RESULTS

### Patient Characteristics

The patient characteristics before treatment are listed in Table 1. Of the 1225 patients (SCLC: 222; adenocarcinoma: 652; squamous cell carcinoma: 194; NSCLC not otherwise specified: 111; large cell carcinoma: 7; others: 39), chemotherapy alone was administered in 884 patients, concurrent chemoradiotherapy in 245, sequential chemoradiotherapy in 43, and thoracic radiotherapy alone in 53 patients. To evaluate the incidence of TRD among the patients who received chemotherapy, radiotherapy, or a combination of these modalities, we included the 43 patients who received sequential chemoradiotherapy into both the chemotherapy-alone group and the thoracic radiotherapy-alone group. Therefore, the patients who received sequential chemoradiotherapy were regarded as having been exposed to the risks of treatment twice. The groups were therefore analyzed as chemotherapy alone in 927 patients, concurrent chemotherapy in 245 patients, and thoracic radiotherapy alone in 96 patients. In these groupings, the percentages of patients enrolled in clinical trials were 62, 53, and 23%, respectively.

### Cumulative Incidence and Causes of TRD

The cumulative incidence and causes of TRD are listed in Table 2. Of the 1225 patients, a total of 23 (1.9%) TRDs occurred. Chemotherapy-related deaths occurred in 7 of 927 (0.8%) patients, including 4 (0.4%) from drug-induced lung injury (gefitinib, $n = 3$ and CBDCA + gemcitabine, $n = 1$), 2 (0.2%) from pneumonia (CBDCA + PTX, $n = 2$), and 1 (0.1%) from unknown cause. The patient who died of unknown cause experienced hemodynamic instability (shock) of unknown etiology within 24 hours of ingestion of the first dose of gefitinib (250 mg). No TRDs from sepsis occurred in this series.

Concurrent chemoradiotherapy-related deaths occurred in 12 of 245 (4.9%) patients, including 11 (4.5%) from radiation pneumonitis and 1 (0.4%) from pneumonia during the last planned cycle of CDDP + VNR. Radiotherapy-

**TABLE 1.** Patient Characteristics

| Characteristics | Chemotherapy Alone[a] ($n = 927$) | Concurrent Chemoradiotherapy ($n = 245$) | Radiotherapy Alone[a] ($n = 96$) |
|---|---|---|---|
| Sex | | | |
| Male | 639 | 201 | 43 |
| Female | 288 | 44 | 53 |
| Age | | | |
| Median (range) | 64 (27–86) | 59 (18–77) | 67 (35–81) |
| Performance status | | | |
| 0–1 | 871 | 245 | 88 |
| 2 | 140 | 0 | 8 |
| 3–4 | 16 | 0 | 0 |
| Stage | | | |
| III | 297 | 235 | 71 |
| IV | 454 | 2 | 17 |
| Postoperative recurrence | 176 | 8 | 8 |
| Histology | | | |
| Non-small cell carcinoma | 760 | 191 | 88 |
| Small cell carcinoma | 167 | 54 | 8 |
| Coincidental lung disease | | | |
| Pulmonary fibrosis | 34 | 1 | 4 |
| Pulmonary emphysema | 69 | 30 | 1 |
| Chemotherapy regimen | | | |
| Platinum + taxane | 368 | 21 | — |
| Platinum + irinotecan | 133 | 1 | — |
| EGFR-TKI | 125 | 0 | — |
| Platinum + etoposide | 95 | 54 | — |
| Platinum + antimetabolite | 85 | 0 | — |
| Platinum + vinca alkaloid | 37 | 168 | — |
| Others | 84 | 1 | — |

[a] Forty-three patients who received sequential chemotherapy followed by radiotherapy are included in the analysis of both the chemotherapy-alone group and radiotherapy-alone group, as described in the text.

EGFR-TKI, epidermal growth factor receptor-tyrosine kinase inhibitor.

**TABLE 2.** Treatment-Related Death and Its Cumulative Incidence

| Characteristics | Chemotherapy Alone[a] ($n$ = 927) | Concurrent Chemoradiotherapy ($n$ = 245) | Radiotherapy Alone[a] ($n$ = 96) |
|---|---|---|---|
| No. of treatment-related deaths | 7 | 12 | 4 |
| Cumulative incidence (%) | 0.8 | 4.9 | 4.2 |
| Sex | | | |
| Male | 5 | 11 | 4 |
| Female | 2 | 1 | 0 |
| Age of patients who died of treatment (yr) | | | |
| Median (range) | 69 (46–77) | 68 (50–77) | 75 (65–77) |
| Causes | | | |
| Treatment-induced lung injury | 4 | 11 | 4 |
| Infectious pneumonia | 2 | 1 | 0 |
| Unknown | 1 | 0 | 0 |
| Chemotherapy regimen | | | |
| Platinum + taxane | 2 | 2 | — |
| EGFR-TKI | 4 | — | — |
| Platinum + antimetabolite | 1 | — | — |
| Platinum + etoposide | 0 | 1 | — |
| Platinum + vinca alkaloid | 0 | 8 | — |
| Others | 0 | 1 | — |

[a] Forty-three patients who received sequential chemotherapy followed by radiotherapy are included in the analysis of both the chemotherapy-alone group and radiotherapy-alone group, as described in the text.

EGFR-TKI, epidermal growth factor receptor-tyrosine kinase inhibitor.

related deaths occurred in 4 of 96 (4.2%) patients: all 4 (4.2%) patients died of radiation pneumonitis.

### Risk Factors for TRD from Chemotherapy

Statistically significant factors identified by the univariate analysis were a performance status of 2 to 4, hypoxia, hypoalbuminemia, hyponatremia, out of clinical trials, and treatment with epidermal growth factor receptor-tyrosine kinase inhibitors (EGFR-TKIs) (Table 3). Although statistically significant, the degrees of hyponatremia in the events were neither clinically significant nor symptomatic for the range of 133 to 137 mEq/L. Pulmonary fibrosis and emphysema were noted in 34 and 69 patients, respectively, among the 927 patients. None of these patients with lung disease died of treatment in this study. Multivariate analysis was not performed because the number of observed events was too small ($n$ = 7).

### Risk Factors for TRD from Concurrent Chemoradiotherapy

None of the factors, except for pulmonary fibrosis, were found to be statistically significant in the univariate analysis, although a trend toward increase in the risk of TRD was observed in patients of advanced age (>70 years) and with lower lobe as the primary tumor site (Table 4). Pulmonary fibrosis appeared to be a statistically significant risk factor for TRD; however, it was excluded from the multivariate analysis because of its limited incidence. Thus, we did not perform multivariate analysis for chemoradiotherapy group, and an analysis of the risk of TRD associated with thoracic radiotherapy alone was not conducted because of the limited number of cases.

## DISCUSSION

We identified a total of 23 TRDs out of the 1225 patients (1.9%) enrolled in this study, which is lower than the rate (2.7%) indicated in a previous report, particularly in relation to the number of TRDs from infections, including pneumonia and sepsis.[1] The reason for the decrease in the incidence of infection-related deaths is likely explained by the infrequent use of triplet regimens when compared with previous studies. Especially, mitomycin-C-containing regimens are regarded as effective regimens in the treatment of lung cancer; however, prolonged neutropenia has been observed with these regimens. Ohe et al.[1] reported that combined mitomycin-C + vindesine + CDDP (MVP regimen) therapy is a risk factor for chemotherapy-related TRD (toxic deaths occurred in 9 of 301 patients; odds ratio [OR] = 9.36, 95% confidence interval [CI] = 1.29–68.0, $p$ = 0.027). In this study, only 35 patients, the majority (89%) of whom were enrolled in a clinical trial, received the MVP regimen. In the past, however, the MVP regimen was widely used as part of practice-based regimens (only 28% recorded under clinical trials). In most cases, patients who were not eligible for clinical trials ended up receiving the MVP regimen. Another reason is the relatively frequent use of EGFR-TKI (in 13.5% of the patients in this study) at present, which does not induce myelosuppression. The reduction in the frequency of TRD might also be explained by a progress in supportive care in the treatments given for cancer treatment toxicities.

This study revealed that drug-induced lung injury was the most frequent cause of TRD in the era of molecular-targeted therapy. Three (75%) of four TRDs from drug-induced lung injury were associated with gefitinib. The re-

**TABLE 3.** Risk Factors for Treatment-Related Death from Chemotherapy

| Factors | No. of Patients | Cumulative Incidence (%) | Univariate Analysis OR (95% CI) | *p* |
|---|---|---|---|---|
| Sex | | | | |
| Female | 288 | 0.8 | 1 | |
| Male | 639 | 0.7 | 1.13 (0.22–5.76) | 0.89 |
| Age | | | | |
| <70 | 689 | 0.6 | 1 | |
| ≥70 | 238 | 1.3 | 2.17 (0.51–9.30) | 0.30 |
| PS | | | | |
| 0–1 | 870 | 0.5 | 1 | |
| 2–4 | 57 | 5.2 | 11.4 (3.53–37.1) | <0.001 |
| Smoking history | | | | |
| No | 271 | 0.4 | 1 | |
| Yes | 656 | 0.9 | 2.49 (0.30–20.8) | 0.40 |
| $PaO_2$ (Torr) | | | | |
| ≥70 | 812 | 0.2 | 1 | |
| <70 | 105 | 4.8 | 19.3 (6.06–61.7) | <0.001 |
| Hemoglobin (g/dl) | | | | |
| ≥13.7 | 371 | 0.5 | 1 | |
| <13.7 | 556 | 0.9 | 1.67 (0.33–8.39) | 0.54 |
| Albumin (g/dl) | | | | |
| ≥3.7 | 663 | 0.3 | 1 | |
| <3.7 | 264 | 1.9 | 6.28 (1.51–26.1) | 0.012 |
| AST (IU/L) | | | | |
| ≤33 | 831 | 0.6 | 1 | |
| >33 | 96 | 2.1 | 3.46 (0.75–16.0) | 0.11 |
| Na (mEq/L) | | | | |
| ≥138 | 819 | 0.1 | 1 | |
| <138 | 108 | 5.6 | 45.5 (13.4–154) | <0.001 |
| Clinical trial | | | | |
| No | 355 | 1.7 | 1 | |
| Yes | 572 | 0.2 | 0.10 (0.58–0.019) | 0.001 |
| Platinum + taxane | | | | |
| No | 559 | 0.9 | 1 | |
| Yes | 368 | 0.5 | 0.61 (0.12–3.14) | 0.55 |
| EGFR-TKIs | | | | |
| No | 802 | 0.4 | 1 | |
| Yes | 125 | 3.2 | 8.56 (2.48–29.5) | 0.001 |
| Platinum + antimetabolite | | | | |
| No | 842 | 0.7 | 1 | |
| Yes | 85 | 1.1 | 1.66 (0.20–13.9) | 0.64 |

Multivariate analysis was not performed because the number of observed events was too small ($n = 7$).

OR, odds ratio; CI, confidence interval; PS, performance status; AST, aspartate transaminase; EGFR-TKIs, epidermal growth factor receptor-tyrosine kinase inhibitors.

**TABLE 4.** Risk Factors for Treatment-Related Death from Concurrent Chemoradiotherapy

| Factors | No. of Patients | Cumulative Incidence (%) | Univariate Analysis OR (95% CI) | *p* |
|---|---|---|---|---|
| Sex | | | | |
| Female | 44 | 2.3 | 1 | |
| Male | 201 | 5.2 | 2.41 (0.35–16.6) | 0.37 |
| Age (yr) | | | | |
| <70 | 221 | 4.1 | 1 | |
| ≥70 | 24 | 12.5 | 3.07 (0.92–10.3) | 0.069 |
| PS | | | | |
| 0 | 114 | 5.3 | 1 | |
| 1 | 131 | 4.6 | 0.87 (0.29–2.62) | 0.81 |
| Smoking history | | | | |
| No | 32 | 3.2 | 1 | |
| Yes | 213 | 5.2 | 1.65 (0.23–11.9) | 0.24 |
| Fibrosis | | | | |
| No | 244 | 4.5 | 1 | |
| Yes | 1 | 100 | 22.2 (5.61–87.8) | <0.001 |
| Emphysema | | | | |
| No | 215 | 4.7 | 1 | |
| Yes | 30 | 6.7 | 1.43 (0.33–6.25) | 0.63 |
| Location of the tumor | | | | |
| Other lobes | 189 | 3.7 | 1 | |
| Lower lobe | 56 | 8.9 | 2.41 (0.82–7.13) | 0.11 |
| Histology | | | | |
| SCLC | 54 | 1.9 | 1 | |
| NSCLC | 191 | 5.8 | 3.11 (0.47–20.6) | 0.24 |
| Hemoglobin (g/dl) | | | | |
| ≥13.7 | 146 | 4.1 | 1 | |
| <13.7 | 99 | 6.1 | 1.48 (0.49–4.42) | 0.48 |
| Albumin (g/dl) | | | | |
| ≥3.7 | 198 | 4.5 | 1 | |
| <3.7 | 47 | 6.4 | 1.40 (0.40–4.99) | 0.6 |
| Na (mEq/L) | | | | |
| ≥138 | 219 | 5.0 | 1 | |
| <138 | 26 | 3.8 | 0.77 (0.11–5.60) | 0.79 |
| Clinical trial | | | | |
| No | 114 | 5.3 | 1 | |
| Yes | 131 | 4.6 | 0.87 (0.29–2.62) | 0.81 |
| Platinum + taxane | | | | |
| No | 224 | 4.5 | 1 | |
| Yes | 21 | 9.5 | 2.25 (0.46–11.0) | 0.32 |
| Platinum + vinca alkaloid | | | | |
| No | 77 | 5.2 | 1 | |
| Yes | 168 | 4.8 | 0.91 (0.27–3.13) | 0.88 |

Multivariate analysis was not performed because only fibrosis was significant in univariate analysis.

OR, odds ratio; CI, confidence interval; PS, performance status; NSCLC, non-small cell lung cancer.

ported risk factors for interstitial lung disease in NSCLC patients treated with gefitinib are male sex, history of smoking, and underlying interstitial pneumonitis.[11] In this study, however, none of these factors were associated with TRD from chemotherapy. Another TRD from drug-induced lung injury occurred in a patient who received gemcitabine, but this patient was also free from underlying pulmonary disease or concomitant use of taxanes, which are reported to be risk factors for gemcitabine-associated interstitial lung disease.[12]

For patients who receive concurrent chemoradiotherapy, we would like to emphasize the previous finding that the

presence of evidence of pulmonary fibrosis on a plain chest x-ray is an extremely strong risk factor for TRD (OR = 166, 95% CI = 8.79–3122, $p < 0.001$).[1] In this study, only one patient with pulmonary fibrosis was identified, and pulmonary fibrosis was not included in the multivariate analysis because of the small number of patients with this factor, because we generally exclude patients with evidence of pulmonary fibrosis on the chest x-ray from consideration of concurrent chemoradiotherapy. This study also suggested that advanced age may be a risk factor for TRD. This is consistent with the results of previous studies.[1,13–15] The association between advanced age and fatal radiation-induced lung injury may be explained by the increased likelihood of these patients developing comorbid lung disease, particularly among patients with a history of heavy tobacco exposure. A meta-analysis of chemoradiotherapy using individual data from 1764 patients with locally advanced NSCLC showed that the benefit of chemoradiotherapy was obtained in elderly patients (≥71 years) as well as in younger patients. However, it might be assumed that patients who are included in such trials are fit patients with minimal comorbidities. In addition, despite the increase in toxicity that accompanied chemoradiotherapy in elderly patients, it seemed that they had disease control and survival rates similar to those of younger patients.[16]

In conclusion, TRD occurred in a total of 1.9% of patients and was caused in the majority of the cases by treatment-related lung injury. This finding is in clear contrast with previous reports which suggested that the principal cause of TRD in lung cancer patients was septic shock.

## ACKNOWLEDGMENTS

*The authors thank Ms. Mika Nagai for her assistance in the preparation of this manuscript.*

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original article

*Annals of Oncology* 22: 2471–2475, 2011
doi:10.1093/annonc/mdq781
Published online 4 March 2011

# Phase II study of nedaplatin and docetaxel in patients with advanced squamous cell carcinoma of the lung

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*Received 16 October 2010; revised 20 December 2010; accepted 21 December 2010*

**Background:** The treatment of squamous cell carcinoma of the lung has not advanced sufficiently. Nedaplatin is a second-generation platinum compound that is active against squamous cell carcinoma of the lung, with a response rate of ~40%.

**Patients and methods:** Eligible patients with advanced squamous cell carcinoma of the lung were treated with docetaxel (60 mg/m$^2$) and nedaplatin (100 mg/m$^2$) administered i.v. on day 1; these doses were determined in an earlier phase I study. The treatment cycles were repeated every 3 weeks. The primary end point was the response rate, and the secondary end points were overall survival, progression-free survival, and toxicity.

**Results:** Twenty-one patients were enrolled. Eighteen of the patients were male, and the median age was 67 years. The objective response rate was 62%. The median progression-free survival time was 7.4 months. The median survival time was 16.1 months, and the 1-year survival rate was 66.7% (95% confidence interval 46.5% to 86.8%). The most common adverse event was neutropenia (grade 3/4, 86%). Non-hematological toxic effects were relatively mild. One patient died of sepsis.

**Conclusions:** Combination chemotherapy with nedaplatin and docetaxel is highly active and has an acceptable toxicity. Further investigation of nedaplatin and docetaxel is warranted.

**Key words:** chemotherapy, docetaxel, nedaplatin, non-small-cell lung cancer, squamous cell carcinoma





## introduction

Lung cancer is the leading cause of cancer-related deaths worldwide [1]. More than half of patients with non-small-cell lung cancer (NSCLC) have advanced disease at the time of their diagnoses, and these patients are candidates for treatment with platinum-based combination chemotherapy [2–4]. A recent meta-analysis showed a significant but modest survival benefit of chemotherapy over supportive care alone, with a 9% increase in overall survival at 1 year [5]. Therefore, advances in treatment of NSCLC are urgently needed.

Historically, histological subtypes have not been used to select appropriate treatments for advanced NSCLC. NSCLC consists mainly of adenocarcinoma, squamous cell carcinoma, and large-cell carcinoma, and these classifications are grouped together as a single entity for therapy. However, recent advances in molecular-targeted agents have changed this paradigm [6–8]. Epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors, such as erlotinib and gefitinib, produce dramatic and sustainable responses when used against adenocarcinoma, especially in the presence of an EGFR mutation [9–11]. Bevacizumab, an mAb for vascular endothelial growth factor, produces an additional survival benefit when combined with carboplatin and paclitaxel [12]. However, fatal hemoptysis has been reported in patients with hilar squamous cell carcinoma; therefore, the use of bevacizumab is now restricted to non-squamous NSCLC [13]. Moreover, the novel multitargeted antifolate pemetrexed shows a greater antitumor activity against adenocarcinoma than against squamous cell carcinoma [14]. As a result of these developments, the survival of patients with adenocarcinoma has been improving; however, that of patients with squamous cell carcinoma has remained the same [15].

Nedaplatin is a second-generation platinum compound that is active against NSCLC, especially squamous cell carcinoma, with a response rate of 40% [16, 17]. An earlier phase I study demonstrated that nedaplatin (100 mg/m$^2$) could be safely administered in combination with docetaxel (60 mg/m$^2$) [18]. Based on the results of this previous study, we conducted a phase II study to evaluate the efficacy and tolerability of nedaplatin and docetaxel.

## patients and methods

### patients and evaluations

The eligibility criteria were as follows: histologically or cytologically proven squamous cell carcinoma of the lung; stage III (unresectable and unfit for

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definitive radiotherapy), stage IV, or recurrent disease after surgery; aged 20–75 years; Eastern Cooperative Oncology Group (ECOG) performance status (PS) of zero or one; chemotherapy naive; measurable lesion according to the RECIST version 1.0; and preserved organ function (white blood cell count ≥ $4.0 \times 10^9$/l, absolute neutrophil count ≥ $2.0 \times 10^9$/l, platelet count ≥ $100 \times 10^9$/l, hemoglobin ≥ 9.5 g/dl, total bilirubin ≤ 1.5 mg/dl (0.3–1.2), aspartate aminotransferase ≤ 100 IU/l (13–33), alanine aminotransferase ≤ 100 IU/l (8–42 for male and 6–27 for female), serum creatinine ≤ 1.5 mg/dl (0.6–1.1 for male and 0.4–0.7 for female), and $PaO_2$ ≥ 60 Torr or oxygen saturation ≥ 94% at ambient air). Patients were excluded if pulmonary fibrosis was visible on a chest radiograph (pulmonary emphysema was allowed), uncontrolled pericardial or pleural effusion that needed immediate drainage was present, symptomatic brain metastasis had occurred, or a concomitant serious illness contraindicating systemic chemotherapy was present. Patients who were pregnant or breast-feeding were also excluded. All the patients provided their written informed consent before enrollment, and the study was approved by the institutional ethics boards of the National Cancer Center.

Clinical status, hematology, biochemistry and chest radiographs were assessed at least every 2 weeks. Disease status was assessed every 2 months. Toxicity was graded using the Common Terminology Criteria for Adverse Events version 3.0.

### protocol treatment

Nedaplatin and docetaxel were administered on day 1 every 3 weeks for up to four cycles. Docetaxel diluted in 250 ml of 5% glucose was administered as a 1-h infusion. Nedaplatin was diluted in >300 ml of normal saline or 5% xylitol and was administered as a 90-min i.v. infusion. Dexamethasone (8 mg), granisetron, and fluids (1000 ml) were also administered i.v.

Dose reduction was required if patients experienced the following toxic effects: grade 3 or more non-hematological toxicity (except for nausea, vomiting, anorexia, hyperglycemia, hyponatremia, mucositis, constipation, rash), grade 4 leukopenia or neutropenia lasting for >4 days, grade 4 thrombocytopenia, febrile neutropenia, >14 days delay of the next chemotherapy cycle.

### design and statistics

The primary objective was the response rate. The secondary objectives were progression-free survival, overall survival, and toxicity. The tumor responses were evaluated by radiologists. The planned sample size was 21, based on an alpha of 0.05, 90% power, H0 = 20% and H1 = 50% [19]. After the accrual of 12 patients, an interim analysis was planned. If only two or fewer responses had occurred, then the study would be stopped early. If seven or fewer responses were observed by the end of the trial, then no further investigation of this combination was deemed as being warranted. This study was registered with University Hospital Medical Information Network-Clinical Trials Registry, available at http://www.umin.ac.jp/ctr/index-j.htm (ID: UMIN000001227).

## results

From August 2006 to January 2009, 21 patients were enrolled. The patient demographics and disease characteristics are summarized in Table 1. All the patients had confirmed squamous cell carcinoma; the median age was 67 years, 18 patients were male, and 16 patients had an ECOG PS of one. Nedaplatin and docetaxel were administered to all the patients. The median number of treatment cycles was 4 (range 1–4). Relative dose intensity for planned cycles of chemotherapy was 77.9% for both docetaxel and nedaplatin.

### efficacy

At the interim analysis, 7 of 13 patients had achieved a partial response. Therefore, the accrual continued up to 21 patients. At the end of the study, the objective response rate was 62% (Figure 1 and Table 2). Figure 2 shows the progression-free survival. The median progression-free survival time was 7.4 months [95% confidence interval (CI) 3.5–11.4 months], and the progression-free survival rate at 1 year was 24.8% (95% CI 4.6% to 44.9%). Figure 3 shows the overall survival. The median overall survival time was 16.1 months (the median follow-up time was 20.9 months for the censored cases), and the overall survival rate at 1 year was 66.7% (95% CI 46.5% to 86.8%). Post-study treatment included radiotherapy (nine for local tumors, two for lymph node metastases, one for bone metastasis, and one for brain metastasis) and systemic chemotherapy (seven with gemcitabine and vinorelbine, two

**Table 1.** Patient characteristics

| | Patients ($n$ = 21) | |
|---|---|---|
| | $n$ | % |
| Age, median (range) (years) | 67 (40–78) | |
| Gender | | |
| Female | 3 | 14 |
| Male | 18 | 86 |
| ECOG performance status | | |
| 0 | 5 | 24 |
| 1 | 16 | 76 |
| Smoking, median (range) (pack-years) | 49 (0–100) | |
| Never smoker | 1 | 5 |
| Former smoker | 6 | 29 |
| Current smoker | 14 | 67 |
| Stage | | |
| III | 11 | 52 |
| IV | 8 | 38 |
| Recurrence | 2 | 10 |

ECOG, Eastern Cooperative Oncology Group.

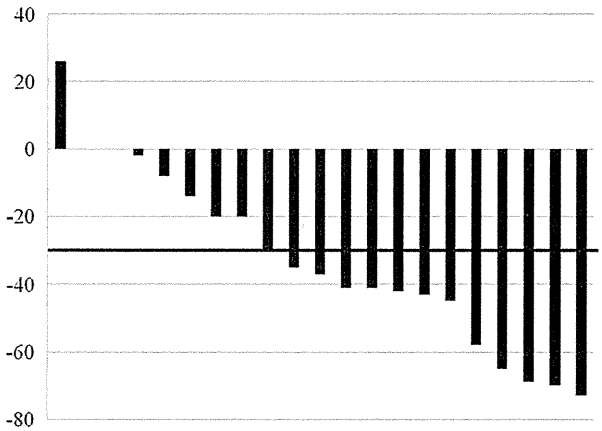


**Figure 1.** Waterfall plots for the degree of tumor shrinkage. Most patients achieved tumor shrinkage. Partial response was observed in 13 patients and the response rate was 62%.

**Table 2.** Objective response (RECIST version 1.0)

| | *n* | % |
|---|---|---|
| Number of patients evaluated | 21 | |
| CR | 0 | 0 |
| PR | 13 | 62 |
| SD | 6 | 29 |
| PD | 1 | 5 |
| NE | 1 | 5 |
| Response rate (95% CI), % | | 62 (39–85) |

CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; NE, not evaluable; CI, confidence interval.

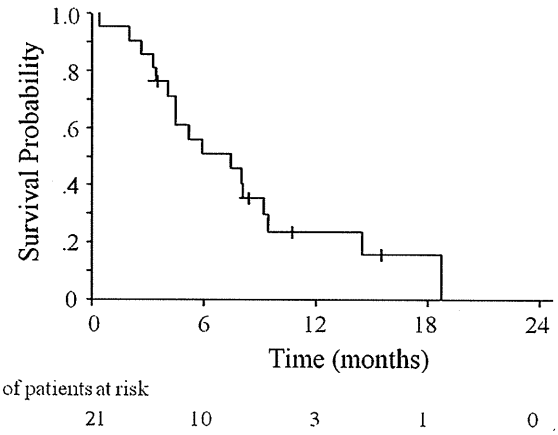


**Figure 2.** Progression-free survival. The median progression-free survival time was 7.4 months (95% CI 3.5–11.4 months), and the progression-free survival rate at 1 year was 24.8% (95% CI 4.6% to 44.9%). CI, confidence interval.

with gemcitabine monotherapy, two with nedaplatin and docetaxel rechallenge, and four others).

### safety

The incidence of treatment-related adverse events is listed in Table 3. Non-hematologic toxicity was generally grade 1 or 2 and consisted primarily of gastrointestinal disorders, with one patient (5%) experiencing grade 3 anorexia and two (10%) experiencing grade 3 nausea. None of the patients experienced grade 3 or higher diarrhea. Grade 3 or higher neutropenia occurred in 86% of the patients, and febrile neutropenia was observed in 24% of the patients. Dose modification was required in seven patients (33%). One patient died of sepsis on treatment day 9 of the first cycle. An autopsy revealed that the patient had previously undiagnosed liver cirrhosis.

## discussion

Nedaplatin is a second-generation platinum compound and is comparable in efficacy with cisplatin against NSCLC, with a reduced renal toxicity. A randomized trial comparing nedaplatin and vindesine with cisplatin and vindesine showed similar survival outcomes, with a median survival time of 8.9 months for nedaplatin–vindesine and 9.1 months for cisplatin–vindesine [20]. However, only 36 of the 136 patients in this series had squamous cell carcinoma of the lung.

Nedaplatin has been combined with gemcitabine, irinotecan, or paclitaxel in patients with NSCLC and with docetaxel mainly in patients with esophageal cancer [21–33].

Nedaplatin is more active against squamous cell carcinoma of the lung than adenocarcinoma as a single agent, based on the results of phase II trials [16, 17]; however, the reason for this difference in the antitumor activity among histological subtypes has not been fully investigated. A preclinical study demonstrated that in squamous carcinoma cells (PC-10), the intracellular concentration of nedaplatin promptly rose and reached a higher concentration than that in adenocarcinoma cells (PC-3), suggesting a higher antitumor activity of nedaplatin against squamous cell carcinoma [34].

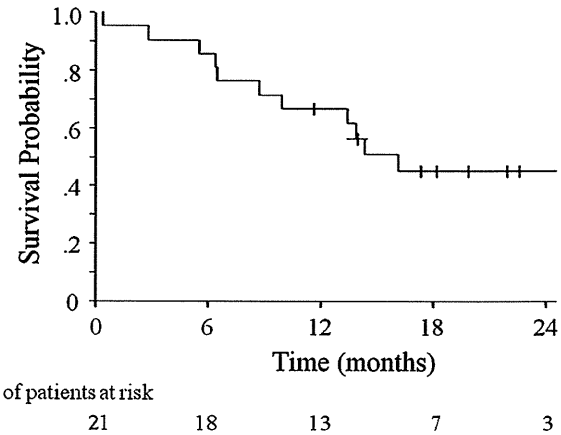


**Figure 3.** Overall survival. The median overall survival time was 16.1 months (the median follow-up time was 20.9 months for the censored cases), and the overall survival rate at 1 year was 66.7% (95% CI 46.5% to 86.8%). CI, confidence interval.

**Table 3.** Adverse events (NCI–CTC version 3.0, $n = 21$)

| Adverse event | Grade 1–2 (%) | Grade 3 (%) | Grade 4 (%) |
|---|---|---|---|
| Leukocytes | 43 | 48 | 10 |
| Neutrophils/granulocytes | 14 | 29 | 57 |
| Hemoglobin | 90 | 5 | 0 |
| Platelets | 86 | 10 | 5 |
| AST | 43 | 0 | 0 |
| ALT | 29 | 0 | 0 |
| Creatinine | 14 | 0 | 0 |
| Febrile neutropenia | 0 | 14 | 10 |
| Anorexia | 67 | 5 | 0 |
| Nausea | 52 | 10 | 0 |
| Vomiting | 24 | 0 | 0 |
| Diarrhea | 48[a] | 0 | 0 |
| Alopecia | 76 | 0 | 0 |

[a]24% grade 2.

NCI–CTC, National Cancer Institute—Common Toxicity Criteria; AST, aspartate aminotransferase; ALT, alanine aminotransferase.

The response rate of 62% in the current study met the criteria for further investigation. Furthermore, the median progression-free survival time of 7.4 months and the median overall survival time of 16.1 months were promising. A selection bias may have caused these survival outcomes. However, the median survival time of 16.1 months is comparable with that of stage III disease treated with definitive chemoradiotherapy and suggested a potential benefit of nedaplatin and docetaxel against squamous cell carcinoma of the lung.

In patients with unresectable squamous cell carcinoma, nedaplatin was also combined with paclitaxel in a phase I trial [35]. The response rate was 55%; however, paclitaxel was reported to be correlated with a shorter progression-free survival time compared with gemcitabine, docetaxel, and vinorelbine based on a meta-analysis [36]. Docetaxel is one of the most promising agents against NSCLC, including squamous cell carcinoma. In the TAX 301 JP trial, the combination of cisplatin and docetaxel showed a statistically significant survival benefit over cisplatin and vindesine [37]. A subgroup analysis showed similar survival benefits for patients with adenocarcinoma and those with squamous cell carcinoma. Therefore, nedaplatin and docetaxel is the most promising combination against squamous cell carcinoma of the lung.

Grade 3 or more neutropenia was observed in 86% of patients in the current study. High incidence of grade 3 or more neutropenia with 84% (74 of 88 patients) treated with docetaxel and cisplatin was also observed in the randomized phase III trial WJOG3405, which compared gefitinib with cisplatin and docetaxel in patients with EGFR-mutant NSCLC in Japan [38]. Moreover, single-agent docetaxel at a dose of 60 mg/m$^2$ resulted in 84.6% (632 of 747) of patients with grade 3 or more neutropenia [39]. In contrast, in the international phase III trial TAX 326 comparing docetaxel plus platinum with vinorelbine plus cisplatin, grade 3 or more neutropenia was observed in 74.5% of patients treated with cisplatin and docetaxel [40]. The reason for this difference is not known. Ethnic difference between the Japanese and the Caucasian is one of the explanations. However, in TAX 301 JP conducted in Japan, grade 3 or more neutropenia in patients treated with cisplatin and docetaxel is 74.1% (112 of 151), which is similar with TAX 326. Neutropenia should be carefully managed and the risk factor of serious neutropenia should be further examined.

In the current study, one patient died of sepsis. The autopsy revealed that the patient was affected by liver cirrhosis, which was been unaware of before treatment. Full dose of docetaxel is not appropriate for patients with impaired liver function and the administration of docetaxel in patients with liver cirrhosis should be avoided [41]. Other toxic effects were relatively mild.

In conclusion, the combination of nedaplatin and docetaxel showed a promising response rate and overall survival time, with acceptable toxic effects. A multicenter phase III trial comparing nedaplatin and docetaxel with cisplatin and docetaxel is currently under way (WJOG 5208L).

## acknowledgements

We deeply appreciate Fumiko Koh for her excellent data management. This study was presented at the World Conference on Lung Cancer 2009 at San Francisco, USA (Abstract PD 3.4.5) and in the Annual Meeting of American Society of Clinical Oncology 2010 at Chicago, USA (Abstract e18014).

## funding

This work was supported in part by a Grant-in-Aid for Cancer Research from the Ministry of Health, Labour and Welfare of Japan.

## disclosure

The authors declare no conflict of interest.

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# Hepatocyte Growth Factor Expression in *EGFR* Mutant Lung Cancer with Intrinsic and Acquired Resistance to Tyrosine Kinase Inhibitors in a Japanese Cohort

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**Introduction:** This study was performed to determine the incidence rates of resistance factors, i.e., high-level hepatocyte growth factor (HGF) expression, epidermal growth factor receptor (EGFR) T790M secondary mutation, and *MET* amplification, in tumors with intrinsic and acquired EGFR tyrosine kinase inhibitor (TKI) resistance in *EGFR* mutant lung cancer.

**Methods:** Ninety-seven specimens from 93 *EGFR* mutant lung cancer patients (23 tumors with acquired resistance from 20 patients, 45 tumors with intrinsic resistance from 44 patients [nonresponders], 29 sensitive tumors from 29 patients) from 11 institutes in Japan were analyzed. HGF expression, *EGFR* T790M secondary mutation, and *MET* amplification were determined by immunohistochemistry, cycleave real-time polymerase chain reaction, and fluorescence in situ hybridization, respectively.

**Results:** High-level HGF expression, *EGFR* T790M secondary mutation, and *MET* amplification were detected in 61, 52, and 9% of tumors with acquired resistance, respectively. High-level HGF expression was detected in 29% of tumors with intrinsic resistance (nonresponders), whereas *EGFR* T790M secondary mutation and *MET* amplification were detected in 0 and 4%, respectively. HGF expression was significantly higher in tumors with acquired resistance than in sensitive tumors ($p < 0.001$, Student's $t$ test). Fifty percent of tumors with acquired resistance showed simultaneous HGF expression with *EGFR* T790M secondary mutation and *MET* amplification.

**Conclusions:** High-level HGF expression was detected more frequently than *EGFR* T790M secondary mutation or *MET* amplification in tumors with intrinsic and acquired EGFR-TKI resistance in *EGFR* mutant lung cancer in Japanese patients. These observations provide a rationale for targeting HGF in EGFR-TKI resistance in *EGFR* mutant lung cancer.

**Key Words:** EGFR-TKI, EGFR mutation, HGF, Acquired resistance, Intrinsic resistance.

(*J Thorac Oncol.* 2011;6: 2011–2017)

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Disclosure: Seiji Yano has received honoraria and research funding from Chugai Pharma.

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ISSN: 1556-0864/11/0612-2011

Epidermal growth factor receptor (EGFR)-activating mutations, in-frame deletion in exon 19, and L858 point mutation in exon 21 are selectively expressed in a population with lung cancer.[1,2] *EGFR*-activating mutations are detected considerably more frequently in nonsmokers, females, adenocarcinomas, and patients from East Asia, including Japan.[3–5] The reversible EGFR tyrosine kinase inhibitors (EGFR-TKIs) gefitinib and erlotinib show dramatic therapeutic efficacy, response rates of 70 to 80%, and significant prolongation of progression-free survival (PFS) compared

with standard first-line cytotoxic chemotherapy in patients with *EGFR* mutant lung cancer.[6–9] However, patients almost always develop acquired resistance to EGFR-TKIs after varying periods.[6,9,10] In addition, 20 to 30% of patients with *EGFR*-activating mutations show intrinsic resistance to EGFR-TKIs.[4] Therefore, intrinsic and acquired resistance to EGFR-TKIs are major problems in management of *EGFR* mutant lung cancer.

Two genetically conferred mechanisms—*EGFR* T790M secondary mutation (T790M secondary mutation)[11,12] and *MET* gene amplification[13]—induce acquired resistance to EGFR-TKIs in *EGFR* mutant lung cancer. In addition, we recently demonstrated the occurrence of hepatocyte growth factor (HGF)-induced resistance.[14] HGF, a ligand of MET,[15] induces EGFR-TKI resistance by activating MET, which restores phosphorylation of downstream MAPK-ERK1/2 and PI3K-Akt pathways,[14] using Gab1 as an adaptor.[16] HGF may be involved in both intrinsic and acquired resistance to EGFR-TKIs in *EGFR* mutant lung cancer.[14]

T790M secondary mutation, *MET* amplification, and high-level HGF expression were detected in clinical specimens from *EGFR* mutant lung cancer patients who acquired resistance to EGFR-TKIs,[11–14,16–18] indicating the clinical relevance of all three resistance mechanisms in lung cancer. Although the number of cases in each study was limited (<30 cases/study), probably because of low availability of biopsy specimens from resistant tumors, *EGFR* T790M secondary mutation and *MET* amplification were estimated to have occurrence rates of 50%[11,12,17,19] and up to 20%,[13,16,17] respectively, in patients showing acquired resistance to EGFR-TKIs. Nevertheless, the incidence of HGF-induced resistance has not been determined. In addition, the incidence rates of these three resistance factors in intrinsic resistance (nonresponders) are unknown.

Here, we performed a large-scale study in 23 tumors with acquired resistance from 20 patients, 45 tumors with intrinsic resistance from 44 patients (nonresponders), and 29 sensitive tumors from 29 patients to determine the incidences of the three resistance factors not only in acquired resistance but also in intrinsic resistance (nonresponders) to EGFR-TKIs in Japanese patients with *EGFR* mutant lung cancer.

## MATERIALS AND METHODS

Patient details are described in the Supplementary information (http://links.lww.com/JTO/A197).

### Definition of Sensitivity to EGFR TKI

Here, tumors with *EGFR* mutation known to be associated with drug sensitivity (i.e., G719X, exon 19 deletion, and L858R) were obtained from patients before or after treatment with a single EGFR-TKI.[9]

Sensitive tumors were defined as those obtained from patients whose tumors showed a decrease in diameter of at least 30% (either documented partial response or complete response) associated with EGFR-TKI treatment in imaging studies (Response Evaluation Criteria in Solid Tumors [RECIST] version 1.0). Tumor specimens were obtained before EGFR-TKI treatment.

Tumors with acquired resistance were defined as described previously.[9] Briefly, cases showing objective clinical benefit from treatment with an EGFR TKI as defined by either documented partial or complete response (RECIST) or significant and durable (>6 months) clinical benefit (stable disease as defined by RECIST) and systemic progression of disease (RECIST), while on continuous treatment with gefitinib or erlotinib within the last 30 days were defined as showing acquired resistance. Tumor specimens were obtained after systemic progression of disease.

As intrinsic resistance (nonresponders) has not been clearly defined, tumors without response to treatment with an EGFR TKI, i.e., either documented stable disease or progressive disease (RECIST), were defined as showing intrinsic resistance (nonresponders). Tumor specimens were obtained either before or after EGFR-TKI treatment.

### Patients

Ninety-seven tumor specimens with EGFR mutations were obtained from 93 lung cancer patients, all of whom provided written informed consent, at 11 institutes in Japan. This study was approved by the Institutional Review Boards of each institute.

Patients' characteristics are shown in Table 1. Eighty-seven patients had adenocarcinomas, one had large cell carcinoma, two had squamous cell carcinoma, two had adenosquamous carcinoma, and one had undifferentiated non-small cell carcinoma. As the first EGFR-TKI, gefitinib and erlotinib were given to 82 and 10 patients, respectively, and the dual inhibitor of EGFR and VEGFR2, vandetanib,[20] was given to 1 patient.

Exon 19 deletion and L858R point mutation in exon 21 of *EGFR* were detected in 40 and 57 of the 97 tumors, respectively (Table 1). Two of these tumors had both exon 19 deletion and L858R point mutation. Two tumors without exon 19 deletion or L858R had G719X. Twenty-three tumors with acquired resistance were obtained from 20 patients after EGFR-TKI treatment. Forty-five tumors with intrinsic resistance (nonresponders) were obtained from 44 patients either before (41 tumors from 41 patients) or after (four tumors from three patients) EGFR-TKI treatment. Twenty-nine sensitive tumors were obtained from 29 patients before EGFR-TKI treatment.

### Immunohistochemistry for HGF

Immunohistochemical staining was conducted on formalin-fixed, paraffin-embedded tissue sections (4 $\mu$m thick) of tumor specimens with microwave antigen retrieval in 0.01 M citrate buffer (pH 6.0). We used rabbit polyclonal antibody against HGF-$\alpha$ (IBL, Gunma, Japan) at 1:20 dilution as a primary antibody and EnVision/HRP Polymer Reagent (Dako, Glostrup, Denmark) and DAB (3,3′-diaminobenzidine tetrahydrochloride) Liquid (Dako) for detection.

### Evaluation of HGF Expression

The percentages of cancer cells with positive cytoplasmic and/or membrane HGF immunoreactivity were evaluated (0 to 100%), and the modal intensity of the positively staining cells on a scale ranged from 0 to 3+ (0, complete